N-terminus of actin is abolished. These results indicate a new communication pathway in myosin. Based on the seesaw model of the relay helix movement, actin pulls the PR loop which directly moves the relay region and accelerates the rate of the power stroke. The kinetic scheme suggests that actin activation is not required directly for the motor functions and motility. The increased ATPase biases the kinetic paths towards the actin bound forms even in weak actin bound states, thus this effect reduces the possibility of futile cycles of the actomyosin ATPase.

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Light Chain Domain Orientation Determined by Time-resolved FRET

David Kast, Sarah E. Blakely, Bengt Svensson, David D. Thomas.
University of Minnesota, Minneapolis, MN, USA.

We have used site-directed time-resolved FRET to determine light-chain domain transitions in expressed *Dictyostelium* full-length myosin S1. Myosin S1 is proposed to undergo large rotations of the lever-arm in response to the nucleotide state. Previous fluorescence experiments (Shih et al., 2000) have shown that S1 adopts two populations of pre-stroke states and the equilibrium between these states is driven by nucleotide. To observe these population changes in detail, and to observe the effect of actin, we have used time-resolved fluorescence resonance energy transfer (TR-FRET) to measure the distribution of distances between the labeled catalytic domain (A250C) and a labeled RLC (M129C). In the present work, we show that the lever arm adopts multiple orientations in solution. In the absence of nucleotide, the light-chain domain probe is at a mean distance of 8.4 nm from the catalytic domain probe. This distance extends to >9.0 nm when flS1 is bound to actin. The addition of ATP gives three roughly equal populations of distances at 3.6, 4.3, and >9.0 nm. ADP.V_i stabilizes the shorter of these populations, indicating that ADP.P_i induces a conformational change of the light-chain domain. This work was supported by grants from NIH (AR32961, AR07612). We thank Igor Negrashov for excellent technical assistance.

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Measuring Myosin Light Chain Domain Orientation in the Pre-Power Stroke AlF_4 States with a Bifunctional Spin Label in Skinned Muscle Fibers

Andrew R. Thompson, Ryan Mello, Christina Yi, David D. Thomas.
University of Minnesota, Minneapolis, MN, USA.

We are using electron paramagnetic resonance and site-directed spin labeling to measure the orientation of the light chain domain of skeletal muscle myosin in pre-power stroke states trapped by the phosphate analog AlF_4. Previous work (Kraft et al. (2005) Proc. Natl. Acad. Sci. USA **102:**13861-66) has shown via single-fiber X-ray diffraction that AlF_4 traps two distinct pre-powerstroke myosin states in activated muscle. The first state (ADP.AlF_4-I) produces a weak actin binding, disordered myosin whereas the second state (ADP.AlF_4-II) produces a strong binding, stereospecific actomyosin complex. This weak-to-strong transition is a clear indication of the initiation of the power stroke before phosphate release, but it does not reveal the state of the light chain domain (LCD), which undergoes a rotation during the power stroke. We measured the orientation of the light chain domain by exchanging the native regulatory light chain (RLC) of skinned rabbit psoas muscle fiber bundles with a Di-Cys mutant RLC labeled with a bifunctional spin label. Our group has shown previously (Thompson and Naber et al. 2008 Biophys J, in press) that a bifunctional methanethiosulfonate spin label binds rigidly to myosin and reports protein orientation accurately. EPR spectra of oriented fibers show that the LCD produces a distinct orientation from rigor and relaxation in the ADP.AlF_4-II state whereas the ADP.AlF_4-I state is indistinguishable from relaxed muscle, supporting the hypothesis that the II state represents a state early in the power stroke with a distinct LCD orientation. This work was supported by NIH (AR32961, AR007612). We thank Bernhard Brenner and Theresia Kraft for guidance.

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Probing The Divalent Cation-binding Region Of The Myosin Regulatory Light Chain During Muscle Contraction Using EPR

Osha Roopnarine, James Hegg.
University of Minnesota, Minneapolis, MN, USA.

We are using electron paramagnetic spectroscopy (EPR) to determine whether the myosin light chain domain (LCD) rotates as a rigid unit during muscle contraction. To accomplish this we are making orientational measurements at selected positions in the LCD using site-directed spin labeling of single-cysteine mutants of RLC. In addition, we are using double-cysteine RLC mutants to measure distance changes using dipolar EPR. We created two RLC mutants that contain a single cysteine (I35C or A55C) in the helices of the helix-loop-helix of the rat ventricular RLC (vRLC) divalent cation-binding site. Starting with each single-cysteine mutant, a second cysteine was added in the opposite helix (I35C/L49C and A55C/A32C) for distance measurements. The four RLC mutants were labeled with the spin probe MTSL and separately reconstituted into rabbit psoas muscle fiber bundles. The EPR spectra of the labeled fiber bundles in rigor were sensitive to muscle fiber orientation, for both I35C- and A55C-RLC mutants, indicating that the spin labels were highly oriented at both sites, making them ideal for measuring orientational changes. For both single-cysteine RLC mutants, myosin in rigor showed two populations of oriented spin labels that became substantially disordered in relaxation, while contraction induced order in a small fraction of the spin labels. The EPR spectra of the fibers with double-cysteine RLC mutants showed that the spin labels were less sensitive to muscle orientation, so they are suitable for distance measurements with myofibrillar preparations at low temperatures. This work is supported by NIH grant AR052360 to OR and by the Minnesota Supercomputing Institute.

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Orthovanadate Slows Kinetics Of The Acto-Myosin Interaction In Skinned Muscle Fibers By Competition Between Myosin-ADP-P_i and Myosin-ADP-V_i Cross-Bridges For Actin Sites

Marco Caremani[1], **Steve Lehman[2]**, Vincenzo Lombardi[1], Marco Linari[1].
[1]University of Florence and CNISM, Florence, Italy, [2]University of California, Berkeley, CA, USA.

Orthovanadate (V_i) is a phosphate analog that has been used to study the relationship between biochemical reactions and structural and mechanical transitions in the cross-bridge cycle, with great success. In solution, V_i binds to myosin with high affinity, forming a stable myosin-ADP-Vi complex. In permeabilized muscle fibers, V_i binds to an actin-myosin-ADP state during cross-bridge cycling, and suppresses isometric tension, isometric stiffness and unloaded shortening velocity. We investigated the effects of V_i on the mechanics and kinetics of the actin-myosin interaction in skinned fibres from rabbit psoas (sarcomere length 2.4 μm, temperature 12 °C). After correction for myofilament compliance, the observed changes in stiffness indicate that the reduction in T_0 isdue to a proportional reduction in the number of myosin cross-bridges attached to actin (Caremani et al., *Biophys J* 480a/2289-Pos, 2007). The effect of [P_i] (range 0-15 mM added [P_i]) on T_0 and the rate constant of force development following a period of unloaded shortening (k_D) in the presence of V_i (0.1 mM) suggest that V_i acts as a competitive inhibitor of P_i for the myosin-ADP state (Caremani et al., *Biophys J* 128a/621-Pos, 2008). Simulations show that the effects of V_i on force, stiffness and rate of force redevelopment, as well as on the actomyosin ATPase (Wilson et al, *Biophys J* 68:216-226,1995) and velocity of unloaded shortening(Chase et al, *J Physiol* 460:231-246, 1993), can be explained with a straightforward modification of the kinetic scheme of Dantzig et al, *J Physiol* 451:247-278, 1992 to include myosin-ADP-V_i heads competing with myosin-ADP- P_i heads for actin sites. Supported by NIH (R01 AR049033.03) and MiUR (Italy).

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Single Molecule Kinetic Measurements Of Non-muscle Myosin IIB Using Optical Tweezers

Attila Nagy[1], Yasuharu Takagi[1], Earl Homsher[1], Davin K.T. Hong[2], Mihaly Kovacs[3], James R. Sellers[1].
[1]NIH, Bethesda, MD, USA, [2]Summit Computers, Washington, DC, USA, [3]Department of Biochemistry, Eotvos University, Budapest, Hungary.

Non-muscle myosin IIB (NMIIB) is a cytoplasmic myosin which is ubiquitously expressed in eukaryotic cells, particularly in the central nervous system and cardiac tissues. Our previous solution kinetic study of the single headed, subfragment-1 (S1) construct of NMIIB showed that this myosin spends a significant amount of its actomyosin ATPase cycle in the strongly bound state. In the presence of actin, the rate of ADP release is slow (~0.35 s^{-1}), comparable to the steady-state ATPase rate (0.13 ± 0.01 s^{-1}). Furthermore, ADP-affinity for NMIIB is the highest reported so far for the myosin super-family (<0.15 μM). These unique kinetic parameters are advantageous for NMIIB, whose function *in vivo* has been shown to be associated to cortical tension generation and maintenance. To study the kinetics of single molecules of NMIIB-S1 constructs interacting with a single actin filament, we used a dual-beam optical tweezer apparatus to perform single molecule kinetic/mechanical studies using the 'three-bead' assay. We measured the lifetimes of unitary actomyosin interactions and determined the actin-detachment kinetics with varying ATP concentrations. Optical trapping results showed that at physiological ATP concentration (1 mM), the rate of detachment of acto-NMIIB-S1 interactions was ~0.3 s^{-1}, similar to the ADP release rate and steady-state ATPase rate reported from solution kinetic studies. Decreasing the ATP concentration (10 μM) did not alter this rate of detachment. Additional single molecule experiments were performed by adding